
Understanding Gene Function and Control in Lignin Formation In Wood

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Tremendous effort has been devoted to developing genetically engineered trees, with the emphasis on reducing lignin quantity to improve woodpulp-production efficiency.

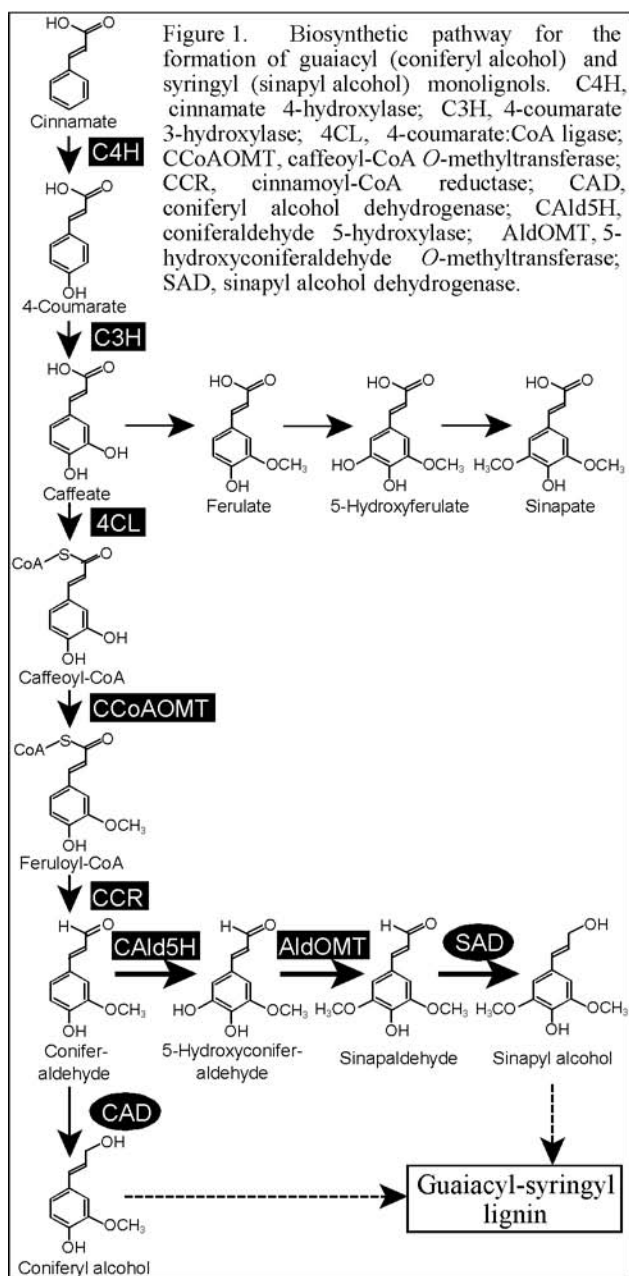
Tremendous effort has been devoted to developing genetically engineered trees, with the emphasis on reducing lignin quantity to improve woodpulp-production efficiency. However, lignin chemical reactivity also is a critical barrier to woodpulp production, as lignin removal from wood is either initiated by chemical degradations or—as in most cases—accomplished entirely through chemical reactions. Thus, the current tree-bio-technology emphasis on low lignin quantity must be expanded to include greater lignin reactivity and, ultimately, a combination of low and reactive lignin traits.

Lignin reactivity depends on the frequency of its structural units, guaiacyl (G) and syringyl (S) monolignols. More syringyl monolignol units, or high S/G lignin monomer ratios, are known to induce high lignin reactivity. For more than 50 years, it has been thought that syringyl monolignol biosynthesis in angiosperms occurs via conversion of caffeate to sinapate via ferulate and 5-hydroxyferulate (Figure 1) (Grisebach, 1981; Grand, 1984; Higuchi, 1985). Based on high-performance liquid chromatography/mass spectrometry (HPLC/MS) characterization of products from reactions of microsomal proteins from lignifying stem xylem of sweetgum (*Liquidambar styraciflua*) with a mixture of four potential 5-hydroxylation substrates—ferulate, feruloyl-CoA, coniferaldehyde and coniferyl alcohol—Osakabe *et al.* (1999) discovered that 5-hydroxyferulate was not synthesized. Instead, the exclusive product from this mixed substrate reaction was 5-hydroxyconiferaldehyde, demonstrating for the first time that a coniferaldehyde 5-hydroxylase (CALd5H) is involved in monolignol biosynthesis, and that ferulate 5-hydroxylase (F5H) may not be (Osakabe *et al.*, 1999).

Subsequently, *Cald5H* cDNAs were cloned from aspen and sweetgum. When coniferaldehyde was incubated with a mixture of CALd5H-containing yeast P450 and *E. coli*-expressed caffeate *O*-methyltransferase (COMT), it was converted to sinapaldehyde via 5-hydroxyconiferaldehyde (Osakabe *et al.*, 1999). Thus, CALd5H catalyzes 5-hydroxylation of coniferaldehyde into 5-hydroxyconiferaldehyde, which in turn is methylated by COMT to sinapaldehyde, supporting the idea of a hydroxylation/methylation flux *in vivo* from guaiacyl to syringyl monolignol biosynthesis via coniferaldehyde (Figure 1). Based on HPLC/MS characterization of the kinetic properties of purified recombinant aspen COMT, Li *et al.* (2000) demonstrated that, indeed, COMT is a 5-hydroxyconiferaldehyde *O*-methyltransferase (AldOMT) that catalyzes methylation of 5-hydroxyconiferaldehyde ($K_m = 2.6 \mu M$) with some affinity for caffeate ($K_m = 75.1 \mu M$) and 5-hydroxyferulate ($K_m = 15.0 \mu M$). However, when a mixture of 5-methylation substrates—caffeate, 5-hydroxyferulate and 5-hydroxyconiferaldehyde—was incubated with recombinant COMT (now designated as AldOMT) or soluble proteins from stem xylem, a complete inhibition of caffeate and 5-hydroxyferulate methylation was observed, while the conversion of 5-hydroxyconiferaldehyde into sinapaldehyde (Figure 1) was conserved (Li *et al.*, 2000). Enzyme inhibition kinetics further showed that 5-hydroxyconiferaldehyde is a competitive inhibitor of AldOMT-catalyzed methylation of both 5-hydroxyferulate and caffeate with K_i values of 0.26 and 2.1 μM , respectively, but 5-hydroxyferulate and caffeate are not effective inhibitors of 5-hydroxyconiferaldehyde methylation (Li *et al.*, 2000). Thus, the presence of CALd5H/AldOMT-mediated coniferaldehyde 5-hydroxylation/methylation eliminates the pathway from caffeate to sinapate via ferulate and 5-hydroxyferulate, and CALd5H/AldOMT diverts the guaiacyl pathway from coniferaldehyde to sinapaldehyde via 5-hydroxyconiferaldehyde to initiate syringyl monolignol biosynthesis (Figure 1).

CAD AND SAD

The CALd5H/AldOMT pathway together with the long-thought coniferyl alcohol dehydrogenase (CAD) function with sinapaldehyde was once believed to lead to the biosynthesis of syringyl monolignol. However, HPLC/MS-based enzyme functional analyses of aspen xylem protein and *E. coli*-expressed recombinant aspen CAD protein demonstrated that CAD is in fact coniferaldehyde- or guaiacyl-specific (Li *et al.*, 2001). This strongly suggests that a discrete sinapyl alcohol dehydrogenase (SAD) is needed for metabolizing the CALd5H/AldOMT product, sinapaldehyde, into sinapyl alcohol, the syringyl monolignol. This discovery led to the isolation of an SAD cDNA from aspen developing xylem (Li *et al.*, 2001). Like the CALd5H/AldOMT-mediated initiation of the syringyl pathway, SAD protein is widely distributed in angiosperms (Li *et al.*, 2000), but SAD as well as CALd5H and AldOMT proteins and their functions are absent from gymnosperms (Li *et al.*, 2001). These results challenge the traditional model of monolignol biosynthesis and suggest that CAD mediates the reduction of coniferaldehyde into guaiacyl monolignol and that SAD along with CALd5H/AldOMT controls the biosynthesis and utilization of sinapaldehyde for syringyl monolignol.



LIGNIN REDUCTION

Biochemical evidence further demonstrated that, in this principle flux, 4-coumarate:CoA ligase (4CL) may limit total lignin accumulation (Hu *et al.*, 1999). 4CL, an enzyme upstream of coniferaldehyde (Figure 1), has been demonstrated to limit lignin accumulation in various plant species. Transgenic aspen trees with downregulated lignin-specific 4CL, Pt4CL1 (Hu *et al.*, 1999), exhibited up to a 45% reduction in lignin, but this did not alter lignin structure with respect to the S/G ratio, as revealed by lignin thioacidolysis (Hu *et al.*, 1999). Two-dimensional heteronuclear single quantum correlation nuclear magnetic resonance (HSQC NMR) further confirmed that the common lignin structural units are all similarly represented in wild-type and lignin-reduced transgenic trees (Hu *et al.*, 1999). Thus, these data provide strong evidence for the absence of any significant branch pathways at caffeate, the preferred 4CL substrate (Hu *et al.*, 1998), that would otherwise divert caffeate metabolism away from the principal phenolic flux (Figure 1) to result in an abnormal type of lignin. We proposed that, with respect to this principal flux, the result of 4CL downregulation is simply the attenuation of metabolite pools downstream of caffeate, limiting the availability of the normal precursors, the monolignols, for lignin polymerization.

Combinatorial gene manipulation had led to 38% to 52% reductions in stem lignin and to 22% to 64% increases in the lignin S/G ratio.

When antisense 4CL and sense CALD5H genes were simultaneously transferred into aspen via *Agrobacterium*, phenotypically normal transgenic trees expressing each one and both of the transgenes were produced (Li *et al.*, 2003). Forty transgenic aspen lines were obtained, of which 37, 40, and 23% harbored antisense Pt4CL, sense LsCALD5H and antisense Pt4CL + sense LsCALD5H gene constructs, respectively, as confirmed by genomic PCR. From each of these three transgenic groups grown in a greenhouse, several trees were randomly selected and harvested at the age of 10 months during the growing season for various characterizations. 4CL-protein levels were drastically reduced in lines harboring only antisense Pt4CL transgene, leading to a 70% to 90% reduction in xylem 4CL enzyme activity, and a 30% to 40% reduction in stem lignin (Table 1). No significant effect on the lignin S/G ratio was found (Table 1). Over-expressing the LsCALD5H gene alone drastically elevated the xylem CALD5H-protein levels, giving rise to a 2.2-2.8-fold increase in xylem CALD5H enzyme activity. As a result, these transgenics exhibited up to a remarkable 2.5-fold increase in the S/G ratio as compared to the control (Table 1). The single CALD5H gene effect had no influence on total lignin accumulation in transgenic trees (Table 1). However, the single-gene effects became additive in transgenics harboring both antisense Pt4CL and sense LsCALD5H genes. Alterations of 4CL- and CALD5H-protein levels in these trees were consistent with changes of the corresponding enzyme activities: 80% to 90% reduction in 4CL and 60% to 110% increase in CALD5H. This

combinatorial gene manipulation had led to 38% to 52% reductions in stem lignin and to 22% to 64% increases in the lignin S/G ratio (Table 1).

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Transgenic trees with reduced lignin exhibited increases in cellulose content—up to a remarkable 30% increase—was observed in antisense-*Pr4CL*/sense-*LsCald5H* transgenic line 141, due to a 52% lignin reduction (Table 1). Consistent with the observation reported by Hu *et al.* (1999), the increased cellulose content together with reduced lignin quantity resulted in a cellulose:lignin ratio of 3 to 5 in the transgenic lines, as opposed to 1.9 in the control (Table 1). The relative abundance of the major hemicellulose component, xylan, was essentially unaffected in all transgenic lines, confirming our previous results (Hu *et al.*, 1999).

*These transgenics are
potentially valuable
lignocellulosic substrates for
woodpulp production.*

CONCLUSION

Lignin reductions in trees can be achieved by antisense *4CL*, technology and over-expression of sense *Cald5H* results in S/G increases. These effects were independent but additive, with plants expressing both transgenes having less lignin, a higher S/G ratio and more cellulose. These transgenics are potentially

Table 1. Chemical compositions in stem wood of control and transgenic aspen

| Plant | Control | 21 | 22 | 23 | 25 | 36 | 32 | 84 | 96 | 94 | 102 | 72 | 74 | 141 | 143 |
|------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Gene integrated | | -4CL | -4CL | -4CL | -4CL | -4CL | +Cald5H | +Cald5H | +Cald5H | +Cald5H | +Cald5H | -4CL | -4CL | -4CL | -4CL |
| -4CL ^a | | | | | | | | | | | | | | | |
| +Cald5H ^b | | | | | | | | | | | | | | | |
| Lignin content (%) ^c | 22.2±0.8 | 16.0±0.6 | 15.3±0.4 | 14.4±0.5 | 13.1±0.3 | 14.9±0.2 | 22.4±0.5 | 21.6±0.4 | 21.1±0.4 | 20.7±0.6 | 19.7±0.4 | 13.7±0.4 | 12.4±0.5 | 10.7±0.4 | 13.2±0.3 |
| Lignin S/G ratio | 2.2 | 2.1 | 2.0 | 2.2 | 2.3 | 2.1 | 4.8 | 4.0 | 5.5 | 4.9 | 3.0 | 3.6 | 3.4 | 2.7 | 3.3 |
| Cellulose content (%) ^d | 41.4±0.4 | 43.1±0.3 | ND | 44.8±0.4 | 47.3±0.5 | ND | 40.0±0.3 | 42.6±0.2 | 44.7±0.1 | 43.4±0.2 | 44.3±0.1 | 49.2±0.3 | 50.9±0.1 | 53.3±0.2 | ND |
| Xylan content (%) ^e | 15.8±0.2 | 16.8±0.3 | ND | 16.1±0.3 | 16.9±0.4 | ND | 16.5±0.4 | 15.3±0.1 | 15.7±0.5 | 15.6±0.5 | 15.2±0.1 | 15.3±0.3 | 14.6±0.2 | 15.4±0.4 | ND |
| Cellulose/lignin ratio | 1.9 | 2.7 | ND | 3.1 | 3.6 | ND | 1.8 | 1.9 | 2.1 | 2.0 | 2.2 | 3.6 | 4.1 | 5.0 | ND |

Values are means ± SE of two to three assays of different samples from each line. ^a(-4CL) and ^b(+Cald5H) denote antisense *4CL* and sense *Cald5H* transgenes, respectively. ^cLignin, ^dcellulose, and ^exylan contents are % of dry wood weight. ND: not determined.

valuable lignocellulosic substrates for woodpulp production. They may not be the ultimate lignocellulosics for bioethanol production, but they are benchmark transgenics and are rich sources of information for understanding cell-wall biosynthesis and thus for further metabolic engineering, allowing the generation of the ultimate raw materials for woodpulp production.

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